

## **EXHIBIT B**

## SYNTHETIC PEPTIDE IMMUNOGENS AS VACCINES

Thomas M. Shinnick, J. Gregor Sutcliffe, Nicola Green, and  
Richard A. Lerner

Research Institute of Scripps Clinic, La Jolla, California 92037

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### INTRODUCTION

#### *Background*

As recently as 3 years ago, a review of synthetic peptide immunogens (9) emphasized that for a peptide to elicit antibodies that could react with an intact protein containing the peptide sequence, it was necessary to construct the peptide in such a way as to reproduce the tertiary conformation of an antigenic site of the intact protein. For example, to make a peptide representing a known hen egg white lysozyme antigenic site comprising residues 64-80 elicit anti-



bodies that bound to intact lysozyme, it was necessary to synthesize the peptide as a covalent loop structure (4, 63, 72). Obviously, such a consideration places restrictions on the usefulness of this approach to generating protein-reactive antibodies, because it requires a great deal of information about the antigenicity and tertiary structure of the protein. The recent explosion in the number and types of applications of the peptide immunogen approach is due in part to the realization that this consideration was an unnecessary restriction. That is, we and others (59, 90) have shown that a peptide corresponding to virtually any region of a protein can elicit antibodies reactive with that protein and it is not necessary to exactly reproduce the tertiary conformation of that region of the protein. These findings raise an interesting point. The antigenicity and immunogenicity of a protein is not the simple sum of the antigenicity and immunogenicity of its pieces (peptides). To investigate this difference, we first illustrate the key points by a brief description of our studies on the hemagglutinin protein of influenza virus, and then, to put the findings in proper perspective, we review the immunogenicity and antigenicity of proteins with an emphasis on results obtained with peptide immunogens. For the purposes of this review, an immunogenic site of a protein is defined as a region of the protein that can induce an immune response against that region. The response can result in either humoral (circulating antibodies) or cellular (T-cell mediated) immunity. This review concentrates on the humoral immune response. An antigenic site of a protein is defined as the region that interacts specifically with such humoral antibodies or immune T cells.

### *Influenza Virus Hemagglutinin*

What is needed to investigate possible correlations between protein structure or antigenic sites and the ability of a peptide to elicit protein-reactive antibodies is a protein whose amino acid sequence and three-dimensional structure have been determined and whose antigenic sites have been identified and mapped. The hemagglutinin of influenza virus not only meets these criteria, its natural immunogenicity is of important medical consequences (65, 103, 105). Intact hemagglutinin, in the course of an immune response to natural infection or purified protein, elicits antibodies that bind to essentially only four antigenic sites, and these sites have been mapped onto the primary and tertiary structures of the hemagglutinin molecule (103, 105). To investigate the immunogenicity of pieces of hemagglutinin, we chose 20 peptides that represented over 75% of the HA1 primary sequence (42). [The hemagglutinin protein is a trimer made up of monomers that contain one copy of each of the HA1 and HA2 polypeptide chains and all the antigenic sites map to the HA1 portion (36, 45, 58, 87).] The peptides were synthesized by solid phase methods (62), and no effort was made to mimic the tertiary structure displayed by the corresponding regions of the protein. Indeed, when mapped to the X-ray crystallographic structure, it can be



seen that the individual peptides participate in diverse protein structures:  $\alpha$ -helices,  $\beta$ -sheets, and random coils. Of the 20 peptides, 18 elicited antibodies that reacted with purified hemagglutinin or intact virus. Importantly, these 18 peptides are not restricted to the four mapped antigenic sites but are scattered throughout the HA1 amino acid sequence. Therefore, the peptides can elicit antibodies not normally elicited during an immune response against the native protein. Hence, the immunogenicity of pieces of a protein (peptides) is different than the immunogenicity of the intact protein. The antigenicity is different as well since none of the 20 synthetic peptides reacted with antibodies elicited by native hemagglutinin. This is not surprising since an intact protein is thought to have only a few, small antigenic sites (discussed below) and antibody binding to these sites is critically dependent on the conformation of the antigenic site. We return to this study throughout the review as a touchstone because the hemagglutinin represents the most studied molecule in terms of its structure, its natural immunogenicity, and the immunogenicity of many of its component peptides, as well as its key role in protection from influenza virus infection.

## PROTEIN ANTIGENICITY AND IMMUNOGENICITY

### *Antigenicity*

The general approach to defining an antigenic site or determinant of a protein that interacts with humoral antibodies to the natural antigen has been to fragment the protein by various enzymatic and chemical techniques and then to ask which fragments retain the ability to interact with the antibodies (reviewed in 15, 16, 34). Once an antigenic region is identified, it can be defined further by comparing the immunologic reactivity of chemically modified or biologically (i.e. mutant) modified proteins or fragments. Finally, chemical synthesis of the antigenic site can be used to identify the exact amino acids and structures or conformations involved in antibody binding.

Three types of antigenic sites have been defined—sequential, continuous, and discontinuous. Sequential determinants are those in which the antibodies recognize the linear sequence of amino acids. Such determinants are rare in globular proteins but can be found in fibrous proteins (34). A continuous antigenic site is a conformationally distinct portion of the protein that is comprised of amino acids in continuous peptide bond linkage. A discontinuous antigenic site is a conformationally distinct portion of the protein that is made up of amino acids not in continuous peptide bond linkage. Such sites arise from the secondary and tertiary folding of proteins. In globular proteins, antigenic sites are small in size, localized on the protein surface, and critically dependent on conformation, i.e. continuous or discontinuous sites. A consideration of lysozyme will illustrate these features.



Hen egg white lysozyme consists of a single polypeptide chain of 129 amino acids and contains four disulfide bonds, each of which is critical for antigenicity (16, 27, 49, 106). Three antigenic sites have been identified and they account for most of the humoral antibody binding capacity of this protein (reviewed in 16). Site one involves residues 5–14 and 125–128, which are linked by a disulfide bond between cysteine residues 6 and 127. Site two involves residues 60–80 and 87–97. These segments are held in close contact by the disulfide bonds between cysteine residues 64 and 80 and residues 76 and 94. Site three involves residues 113–116 and 30–34, which are linked by the disulfide bond between cysteine residues 30 and 115. To define precisely which amino acids are involved in antibody binding, Atassi and co-workers (16, 18, 44) chemically synthesized oligopeptides that attempted to reproduce the conformation of the surface of the protein. That is, from the X-ray crystallographic structure of lysozyme, they determined which residues were exposed on the surface and their relative positions. Then, by incorporating glycine “spacer” residues, they were able to generate the proper relative position of the exposed residues. An analysis of the ability of such “surface-simulated” peptides to bind antibodies elicited by native lysozyme revealed that the critical amino acids for antibody binding for antigenic site one are Arg<sub>125</sub>, Arg<sub>5</sub>, Glu<sub>7</sub>, Arg<sub>14</sub>, and Lys<sub>13</sub>; for site two they are Trp<sub>62</sub>, Lys<sub>96</sub>, Lys<sub>97</sub>, Asn<sub>93</sub>, Thr<sub>89</sub>, and Asp<sub>87</sub>; and for site three they are Lys<sub>116</sub>, Asn<sub>113</sub>, Arg<sub>114</sub>, Phe<sub>34</sub>, and Lys<sub>33</sub>. Each of these sets of critical amino acids occupies a spatially continuous portion of the protein surface, yet each is sequentially discontinuous. Thus, these antigenic sites are small, located on the surface, dependent on conformation, and discontinuous.

Arnon and co-workers (7, 12, 14, 63, 72, 93) have identified a fourth antigenic site of lysozyme. A stable loop structure corresponding to residues 57–83 has been isolated by pepsin cleavage followed by reduction and re-oxidation of the disulfide bonds (14). The loop (residues 64–82) has also been chemically synthesized (12). When the disulfide bond between cysteine residues 64 and 80 is intact, the loop peptide can bind 4–15% of the antibodies elicited by native lysozyme (12, 14, 72, 93). The loop peptide loses its antigenicity if it is opened by either proteolytic cleavage or reduction and carboxymethylation (12, 14, 93). Within this loop, the arginine residue at position 68 strongly influences antibody binding and hence has been designated a “critical” residue for antigenicity (46). The above studies may have missed this site and critical residue because none of the fragments they assayed for antibody binding contained an intact loop structure. Since the amino acids in this loop are sequentially continuous, the site represents a conformationally dependent continuous antigenic site.

Another procedure for identifying the amino acid residues that are important in antibody binding is to correlate the antigenicity of highly conserved proteins (i.e. greater than 90% amino acid sequence homology) with their amino acid

sequences (101). For example, turkey lysozyme and chicken lysozyme differ by only seven amino acids (56). The sequences are identical at the above identified 17 "critical" residues, yet the immunoreactivities of the proteins are easily distinguished in sensitive immunologic assays, such as the microcomplement fixation technique (28, 76). Therefore, one or more of the seven different residues are involved in antibody binding. Also, by screening five monoclonal antibody preparations elicited by chicken lysozyme for reactivity with a battery of avian lysozymes, Smith-Gill et al (88) showed that each monoclonal antibody preparation had a unique pattern of binding specificity and that the binding of four of the five antibodies was influenced by amino acids clearly outside the four antigenic sites described above.

Two hypotheses have been forwarded to explain the antigenicity of lysozyme (17, 20, 75, 101). Its antigenic sites are strongly influenced by conformation and hence the differences among the immunologic reactivities of the various avian lysozymes could reflect subtle changes in the structure of the antigenic sites through long-range conformational interaction (17, 101). This is apparently the case in studies on sperm-whale myoglobin, in which the heme group is replaced with various metalloporphyrins, causing changes in the myoglobin conformation as measured by optical rotatory dispersion, circular dichroism, and analytical ultra-centrifugation and a decrease in reactivity with antibodies elicited by native myoglobin (5, 16, 26, 34).

The second hypothesis is that lysozyme actually has many sites that can bind antibody, but in the course of a normal immune response, humoral antibodies are produced against only a few antigenic sites (29, 46, 47, 75, 104). The results with the antibodies elicited by the influenza virus hemagglutinin peptides support this model in that antibodies can bind to many regions of the hemagglutinin protein. On the other hand, antibodies elicited by native hemagglutinin or intact virus failed to bind to any of our 20 peptides. This suggests that either the normal immune response recognizes antigenic sites not mimicked by peptides or antibodies are made against a few immunodominant regions or both. The immunodominancy of a few regions would produce an antiserum in which the majority of the antibodies are directed against a few antigenic sites. The remainder of the antibody-binding sites of the protein may be recognized by antibodies representing only a small fraction of the total population. In this light, Atassi and co-workers (16) identified the antigenic sites corresponding to the immunodominant sites of lysozyme. Arnon, Smith-Gill, and co-workers (12, 14, 46, 88, 93) have identified antigenic sites that may correspond to the immunorecessive sites of lysozyme. What is not apparent from such studies is why the bulk reactivity of the polyclonal serum is against a few sites, whereas monoclonal antibodies with other specificities are encountered at a high frequency. Regardless of which hypothesis is correct, the fact that most antibodies bind to only a few sites must be reflected in the immunogenicity of a protein.



### *Protein Immunogenicity*

Our understanding of the immunogenicity of proteins is biased by the current models for the immune response (reviewed in 50, 84, 86, 96). The relevant model concerns the immune response to a hapten-carrier complex, because a given immunogenic site of a protein can be viewed as a hapten attached to the remainder of the protein (carrier). A strong humoral immune response (production of circulating antibodies) directed against the hapten is thought to require the stimulation of two cell types: Specific pre-B lymphocytes must be stimulated by the binding of the hapten to their immunoglobulin receptors. These cells mature into the antibody secreting lymphocytes. A strong response also requires the stimulation of helper T lymphocytes by the interaction of the carrier portion of the hapten-carrier complex with the appropriate T-lymphocyte cell surface receptor. Thus, an effective immunogen will have two spatially distinct immunogenic sites—one for B cell interaction and one for T cell interaction.

The immunogenicity of a hapten is not completely understood, but it is known to be influenced by several factors such as accessibility to binding by the B-cell surface receptors, degree of foreignness, ability of the carrier to stimulate helper T-cell production or activity, stability of the immunogenic site with respect to macrophage degradation and presentation to the immune system, size of the reacting B- and T-cell populations, etc. A brief discussion of some of these factors may shed light on the immunodominance of certain sites (see 11, 50, 84, 96 for more comprehensive reviews).

Obviously, an immunodominant site must be quite different from any determinant that corresponds to self; otherwise the immune system would not produce antibodies due to tolerance considerations. Also, since the initial step in stimulation of pre-B lymphocytes is binding of the hapten to the immunoglobulin receptor on the pre-B cell, the hapten must be readily accessible on the protein surface. Such is the case for the mapped antigen sites of sperm-whale myoglobin and lysozyme. (These mapped antigenic sites are considered to be immunodominant because the majority of antibody elicited by the native protein binds to these sites.) For each protein, the antigenic sites were identified as described above and then were mapped to the protein's crystal structure (15, 16). In all cases, the antigenic sites occupy highly exposed regions on the protein surface. For example, the five mapped antigenic sites of sperm-whale myoglobin occupy corners or bends in the polypeptide chain between helical segments. An analysis of a space-filling model of myoglobin revealed that these "corners" tend to stick out from the rest of the molecule and hence would be readily accessible for receptor binding. A corollary of this is that immunorecessive regions would be less exposed, and in the extreme, buried residues might be immunologically silent (81).



Besides B-lymphocyte interaction, helper T cells must be produced to generate a strong immune response against most antigens. That is, while a hapten reacts with the B-cell receptor, the carrier presents an inducer of helper T cells to the immune system. The T-cell interaction is influenced by the nature of the site that binds to the T-cell receptor, the fate of that site during macrophage presentation, and the relative positions of the B-cell and T-cell binding sites in the hapten-carrier complex. Thus, an immunodominant site for B-cell stimulation must also reflect an appropriately positioned strong inducer of helper T cells. Immunorecessive sites may involve the absence of a strong inducer of helper T cells or the induction of suppressor T cells that act to suppress the production of mature B cells.

From the above discussion it is not surprising that immunodominant, immunorecessive, and even immunosilent regions of a protein play roles in the immune response to a native protein. But why do peptides elicit antibodies that react with the immunorecessive or possibly immunosilent regions. Two key facts are that the peptide is usually coupled to a carrier protein such that it is readily accessible for binding to B cells, and that the carrier protein is selected because it elicits a strong immune response, i.e. is a strong inducer of helper T cells. We would therefore expect that strong immune responses should be made against such carrier-linked peptides. The next section discusses the ability of the peptides to elicit antibodies that will bind to proteins.

## PEPTIDE IMMUNOGENICITY

### *Theoretical Considerations*

The ability of a peptide to elicit antibodies reactive with a native protein must reflect the ability of the peptide to mimic the corresponding region of the native protein. Three lines of evidence support this concept. First, the immunogenicity and antigenicity of a full-length protein are critically dependent on conformation, suggesting that the immunogenicity and antigenicity of individual regions might also be conformation dependent (6, 15, 16, 34). Second, peptides that are constructed as to mimic the protein's conformation elicit protein-reactive antibodies and can react with antibodies elicited by the native protein (12, 14, 46, 93). Third, peptides that are constructed as to not mimic the appropriate conformation (yet have the same amino acid sequence) can neither elicit protein-reactive antibodies nor react with antibodies to the native protein (34, 93). What about peptides whose conformations have not been artificially constrained? From the influenza virus hemagglutinin studies described above, we know that 18 of 20 such peptides did elicit antibodies reactive with the conformationally native protein (42). Hence, at some point, the peptides must



have mimicked the conformation of the corresponding region of the native protein.

A simple explanation for the ability of a peptide to elicit protein reactive antibodies is to suggest that in solution a peptide can adopt a variety of interchangeable conformations, some of which mimic those found in the native protein (35). Since occasional proteolytic fragments of myoglobin can inhibit the binding of antibodies elicited by native myoglobin to the native protein (a process that has been shown for these cases to be critically dependent on the conformation of the antigenic site), it seems likely that at least a fraction of each fragment is in a conformation that mimics that found in the native protein (19, 35, 92). However, only a small fraction of the fragment can be in the proper conformation since physical measurements on these peptides in solution revealed that the peptides lack any ordered structure (19, 35, 92). Indeed, a mathematical analysis of the possible conformations of an inhibitory peptide fragment of staphylococcal nuclease suggests that at any one time the fraction of peptide in a given conformation will be too small to detect by physical measurements (80). If the random adoption of the "correct" conformation is the only consideration in the ability of peptides to elicit protein-reactive antibodies, then one would predict that shorter peptides (5–7 amino acids) would be better at eliciting such antibodies than longer peptides (10–15 amino acids), because they could adopt fewer conformations and thus the "correct" conformation would be mimicked more often. However, our experience is that the longer peptides are as good as, if not better than, shorter peptides in eliciting protein-reactive antibodies (42, 60). Furthermore, the conformation of a peptide is not a simple reflection of random orientation of the various atoms. Some relatively short-range interactions can influence the frequency with which some conformations are adopted, some being preferable to others. Obviously, steric hindrances will prohibit some conformations. Also, the side chains of individual amino acids can interact and stabilize or destabilize certain conformations. Proline residues form an imide rather than amide peptide bond and hence define the orientation of the peptide bond backbone as either *cis* or *trans*. In the *cis* orientation, the peptide chain folds back on itself and brings residues on both sides of the proline residue into close contact. These residues could participate in electrostatic or hydrophobic interactions to stabilize particular peptide conformations. Such a stabilized conformation may represent the conformation found in the native protein since the same kinds of short-range interactions play important roles in protein folding. It is thought that regions or domains of a protein first fold into a stable local conformation and then the folded domains assemble to form the final tertiary structure of the protein (reviewed in 100). For example, the folding of myoglobin is thought to occur by seven individual segments first forming  $\alpha$ -helices, and then the individual  $\alpha$ -helices assemble into the final compact globular structure (78). If the interac-



tions involved in the correct folding of protein domains occur within segments represented by peptides, then one would expect that the relatively short peptide might often adopt the conformation seen in the native protein.

These considerations suggest that a peptide in solution can adopt many conformations, but what kinds of conformations will elicit antibodies that cross-react with the native protein? Since two protein-protein interactions are involved, it is probably only necessary for the effective peptide conformation to resemble that of the protein. In the first protein-protein interaction, the peptide must bind to the immunoglobulin receptor on the surface of the appropriate B cell. In this case, the appropriate B cell is one that produces an antibody species that can bind to the native protein. Obviously, the peptide that exactly mimics the native conformation will bind with high affinity to the appropriate receptor. Conformations resembling the native one might bind with lower affinity and once in the antibody binding site may be locked into the high affinity conformation. Thus, triggering of an appropriate B cell may be accomplished by several of the possible peptide conformations. A second protein-protein reaction involves the binding of antibody to the native protein. Once again, antibodies directed against the appropriate conformation will bind with high affinity to the protein. Also, proteins are not static structures (reviewed in 43) and individual regions can adopt a limited number of conformations. Thus, native conformation actually refers to the limited number of conformations adoptable by a given portion of the protein. Antibodies directed against these alternative native conformations can also bind to the protein. However, since some of these conformations appear less often than others, higher serum concentrations may be required to detect binding to the conformations, and hence the protein. Additionally, since the antibody-antigen reaction obeys mass action laws, the binding to native protein by low affinity antibodies such as those directed against similar conformations can be detected under certain conditions such as high concentrations of antibody and antigen. Therefore, to elicit protein-reactive antibodies, it is probably not necessary to exactly reproduce the native conformation, since in most cases a peptide can adopt the suitable conformation in solution without the experimenter having introduced any restraints. Of course, for a region whose conformation is highly constrained in the native protein such as the loop of lysozyme, it may be necessary to mimic conformation since a peptide would rarely adopt such a constrained conformation.

### *Practical Considerations*

Since not all peptides elicit protein-reactive antibodies, what are the characteristics of an effective peptide immunogen? Peptides corresponding to the amino or carboxyl terminus often elicit protein-reactive antibodies (90). This may reflect that these regions are often immunogenic and antigenic in native

proteins, and a peptide coupled to carrier may closely resemble a terminal region of a native protein. Such peptide immunogens have been successfully used to elicit protein-reactive antibodies for the coat protein of tobacco mosaic virus (3, 4), p30 of mammalian retroviruses (69), *env* polyprotein of Moloney murine leukemia virus (91), middle-T antigen of simian virus 40 (98) and polyoma viruses (97), surface antigen of hepatitis B virus (60), hemagglutinin of influenza virus (42), and transforming proteins of Moloney (71), Feline (83), Rous (68), and simian (79) sarcoma viruses.

Internal peptides can also be effective immunogens. Of the 18 peptides that elicited protein-reactive antibodies in the influenza virus hemagglutinin study, 15 were internal fragments. These peptides represent several different secondary and tertiary structures present in the hemagglutinin molecule,  $\alpha$ -helix,  $\beta$ -sheet, random coil, etc. The only apparent structural feature necessary for eliciting protein-reactive antibodies is that the peptides represent a region of the protein that is accessible to antibodies, i.e. a region on the protein surface. From this study (42) and a study of 13 peptides corresponding to portions of the hepatitis B virus surface antigen (60), we devised a set of guidelines for the selection of peptides to be used as immunogens (60, 90). First, the peptide should contain sufficient numbers of polar or charged amino acids to render it soluble in aqueous solutions and hence easy to handle. By selecting peptides containing such residues, it seems that one is also selecting peptides that tend to be on the surface of the native protein—a requirement for the region to be accessible to antibodies when in the native, full-length protein. Second, peptides should be longer than pentamers because shorter peptides tend not to be immunogenic, and it appears that peptides of length greater than 10 residues have a high probability of eliciting antibodies reactive with the native protein. Third, peptides containing proline residues in addition to polar or charged residues routinely elicit antibodies that react with the conformationally native protein. This may be because prolines are typically found at exposed "corners" or bends in the polypeptide chain and such regions are often accessible to antibodies, or, as discussed above, proline residues may enhance mimicking of the native conformation. With these three criteria, more than 90% of the peptides we have tested have elicited antibodies that react with the native protein.

As is true for small molecules in general, most peptides must be coupled to a carrier protein to elicit a strong immune response (50). Thus, the peptide should contain a residue through which it can be coupled. We routinely couple through cysteine residues using *m*-malimidobenzoyl-*N*-hydroxysuccinimide (91) or free amino groups using glutaraldehyde (98). The cysteine residue need not be present in the primary amino acid sequence but can be added to either the amino or carboxyl end. One caution with glutaraldehyde coupling is that it modifies the free amino group of lysine residues and hence may modify a critical antigenic residue. The choice of carrier protein does not appear to be critical—



keyhole limpet hemocyanin, bovine serum albumin, thyroglobulin, edestin, and synthetic carriers have been used with success. Of course, the ultimate use of the antibodies does place restrictions on the carrier. For example, one would not couple a peptide to bovine serum albumin and use the resulting antibodies to assay cells grown in tissue culture medium containing fetal calf serum. Some peptides have been excellent immunogens without coupling to carrier, for instance, a 24-residue peptide of influenza hemagglutinin (42). Such large peptides may contain both a T-cell and B-cell determinant.

Immunization protocols do not appear to play a role in determining if a peptide will elicit an anti-native protein response but rather influence antibody titer. In our hands, most peptides elicit a good immune response with the following immunization schedule. On day 1, rabbits are injected in the footpads with 200  $\mu$ g of peptide (usually coupled to carrier) in complete Freund's adjuvant. On days 14 and 28, the rabbits are injected intraperitoneally with 200  $\mu$ g of peptide in incomplete Freund's adjuvant. Sera are collected on day 35. Occasionally, a peptide will require several booster injections to generate a reasonably titered antiserum. Interestingly, repeating the immunization schedule with the peptide coupled to a different carrier has given five- to ten-fold increases in antibody titer for certain carrier combinations.

## MEDICAL APPLICATIONS OF SYNTHETIC IMMUNOGENS

### *Detection of Disease*

As we have seen, synthetic peptide immunogens can elicit antibodies that bind to proteins. How can this fact be exploited in the detection, treatment, or prevention of disease? Obviously, if one has an antibody that specifically reacts with a protein, one can develop an assay to detect that protein, such as a radioimmune or solid-phase enzyme-linked immunosorbent assay (ELISA). For example, antibodies against the carcinoembryonic antigen of the colon are used in various radioimmune assays to detect cancer, because this antigen is present on the surface of many types of cancerous cells and in the sera of patients with certain types of cancer (38, 41, 54, 94). Arnon et al (10) have synthesized a peptide corresponding to the amino terminal 11 residues of the carcinoembryonic antigen. Antibodies elicited with this peptide reacted with a crude preparation of antigen. Also, antibodies against the intact protein react with the peptide. These reactivities have been used to produce an immunoassay to detect serum carcinoembryonic antigen levels (13).

### *Passive Vaccination*

The ability of antibodies to inhibit enzymatic activity (8) raises the possibility that peptide-elicited antibodies could be used in a passive immunization therapy. For example, the treatment for exposure to various toxins such as the one



in rattlesnake venom is to inject an antitoxin that contains antibodies that will bind to and inactivate the toxin. Audibert et al (21) have raised antisera in guinea pigs against a tetradecapeptide corresponding to residues 188–201 of the 62,000-dalton polypeptide chain of diphtheria toxin. These antibodies can bind to purified toxin and, when mixed with toxin in vitro prior to intradermal injection into guinea pigs, can prevent the typical dermonecrotic reaction. Such success in vitro provides promise for the development of a passive vaccine that will work in vivo.

### *Vaccines*

An ideal vaccine would have several key features. First, it must confer effective, reliable immunity against the targeted pathogenic organism. Second, it should not produce any unwanted side effects such as a mild form of the disease. Finally, for economic reasons, the vaccine should be inexpensive, safely manufactured and easily disseminated. Synthetic peptide immunogens may have these characteristics, but first let us consider the antiviral vaccines currently used.

Current antiviral vaccines consist of either killed (e.g. Salk poliovirus vaccine) or attenuated (e.g. Sabin poliovirus vaccine) virus. Both attenuated and killed virus vaccines have several drawbacks. First, virus must be grown in large cultures to allow isolation of sufficient antigenic mass for the immunization protocols. Such cultures generally take the form of virus-infected cell cultures (e.g. poliovirus or adenovirus) or virus-infected embryonated chicken eggs (e.g. influenza virus). But not all viruses can be propagated outside the target organism. For example, a major stumbling block to the production of an anti-hepatitis B vaccine is that this virus grows only in higher primates (64, 70). Second, such laboratory cultures are large reservoirs of the pathogenic organism, that require stringent containment procedures to prevent accidental release, a sizeable problem given the necessary large scale of vaccine production. Also, such large numbers of viruses are produced that variants are often generated. Indeed, large, natural reservoirs are the sources of the variants that cause the recurring epidemics of influenza (70, 99). Third, because with such large numbers of virus random mutation could generate a pathogenic virus from the original attenuated strain, the cultures must either be shown to contain only attenuated virus or the virus must be inactivated. Improper or incomplete inactivation of a killed foot-and-mouth virus vaccine has been shown to be the cause of recent outbreaks of foot-and-mouth disease in Europe (24, 51). Fourth, many virus preparations are unstable and require special handling such as refrigeration. Such handling requirements, of course, present barriers to the worldwide distribution of a vaccine, especially in the tropics and underdeveloped areas. Finally, because the viruses were grown in cell culture or eggs, the virus preparations contain many contaminating substances. The contamination of some of the early batches of the Salk poliovirus vaccine with simian

virus 40 is a well-known example. Obviously, such contaminants can cause undesired side effects.

Recently, recombinant DNA technology (30, 52) has been employed to generate immunogens for use as vaccines to circumvent some of these concerns. In brief, a gene encoding the target protein of neutralizing antibodies is cloned into a special vector that usually carries all the transcription and translation signal sequences necessary to express the protein in bacteria or yeast. Three drawbacks to this procedure are that each gene typically requires a unique set of manipulations to get it expressed in an *Escherichia coli* or yeast cell; the expressed protein is too often unstable in the host cell; and finally, what does one use as the immunogen? If one uses a crude extract of the *E. coli* or yeast cells or, if the protein is secreted, the extracellular fluid, then one has the same problem as described above, namely an impure vaccine with unknown or uncharacterized contaminants. Furthermore, the immunogenicity of a hybrid protein, or even an exact replicate of the natural antigen, may not be adequate for a vaccine. For example, genetically engineered VP1 of foot-and-mouth disease virus is a poor immunogen (52).

Synthetic peptide immunogens overcome many of these problems because they do not require the propagation of the pathogenic organism, are chemically pure, and are stable under normal conditions such that storage and handling are simple procedures. But the key question remains whether a synthetic peptide vaccine can confer immunity.

The precise mechanism of antibody-mediated virus neutralization is unclear. However, antibody binding to virus is essential (61). Synthetic immunogens can elicit antibodies that react with viral surface proteins, but can they neutralize virus? Anderer (3, 4) used the carboxyl terminal hexapeptide of tobacco mosaic virus to produce antibodies that could bind to purified coat protein and the virus particle. Mixing these antibodies with tobacco mosaic virus in vitro prior to application to tobacco leaves resulted in the neutralization of the ability of the virus to induce lesions on the leaves. This in vitro neutralization of a plant virus suggests that synthetic peptide immunogens might elicit protection in vivo in vertebrates against animal pathogens.

To study the role of antibodies to peptides in virus neutralization in vitro and in vivo, we extended our study of the influenza A virus and initiated studies on the foot-and-mouth disease virus. As described above, we analyzed 20 peptides to the HA1 molecule of the influenza A virus X-47 and found that 18 of the 20 peptides produced antibodies that bound to intact virus (42). An additional six peptides have been studied that represent a portion of antigenic site C, the HA1-HA2 junction, and the amino terminus of HA2 (2). All six peptides elicited antibodies that reacted with intact virus.

Antibodies to the 26 peptides were assayed for their ability to neutralize virus in vitro (2). Antisera and X-47 virus were mixed and incubated at 37° for 1 hr. The mixture was then added to monolayers of Maden-Darby canine kidney



epithelial cells. After 2 days, the cells were observed for the cytopathic effect associated with influenza A virus. Of the 26 anti-peptide sera, 13 neutralized X-47 virus in this assay. The peptides that elicited these *in vitro* neutralizing antibodies clustered about cysteine residues involved in disulfide bonds that are critical for the conformation of the hemagglutinin and at the HA1-HA2 junction. The cleavage of the HA1-HA2 junction to generate the carboxyl terminus of HA1 and the amino terminus of HA2 appears to be functionally important in the fusion of virus to cell (53, 58).

The 13 positive antisera were also assayed for their ability to neutralize other influenza A subtype H<sub>3</sub> viruses, influenza A subtype H<sub>1</sub> viruses, and an influenza B virus. Antisera raised against intact X-47 (H<sub>3</sub> subtype) virus neutralized the H<sub>3</sub> subtype viruses, and, very weakly, one of the H<sub>1</sub> subtype viruses. On the other hand, antibodies to peptides that correspond to amino acid sequences common to the hemagglutinins of all the viruses neutralized all of them. Thus, for *in vitro* neutralization, one can generate either subtype-specific vaccines or a vaccine that could give broad-range protection. Obviously, if this holds true *in vivo*, then one could produce an anti-influenza virus vaccine that may not become outdated with each new variant of influenza virus. The key to such a vaccine lies in the common amino acid sequences that may reflect that the particular amino acids are required for the function of the hemagglutinin in cell attachment and virus-cell fusion. This is potentially quite important and illustrates an aspect of synthetic peptide immunogens. Neither influenza virus nor intact hemagglutinin elicits a cross-subtype neutralizing response because most of the antibodies are directed against the four immunodominant sites, and it is at these sites that the subtype hemagglutinin sequences vary the most—as expected for an organism attempting to escape a neutralizing immune response. A synthetic peptide can therefore elicit an important set of neutralizing antibodies not available by classical techniques.

To test the influenza peptides as vaccines, we immunized mice with mixtures of the original 20 peptides. After an immune response was detected, the mice were challenged with 80 lethal doses of mouse-adapted X-47 virus. Several peptide combinations were able to protect the mice from death. So far only two of the five peptides tested individually were able to confer protective immunity. In similar experiments with a peptide corresponding to residues 91–108 of HA1, Muller et al (67) were able to neutralize the cytopathic effect *in vitro* and were able to inhibit virus growth *in vivo* by immunization with that peptide. These experiments demonstrate that synthetic peptide immunogens can elicit the protective immunity required of a successful vaccine.

Our other model system to test the feasibility of synthetic peptide vaccines is the foot-and-mouth disease virus (FMDV). This picornavirus contains about 60 copies of each of the four virus proteins per virion (32). Viral protein 1 (VP1) is thought to be the major target of a neutralizing immune response (89,



102). Seven peptides corresponding to portions of VP1 were synthesized and used as immunogens in rabbits (25). Antisera to three of these peptides were capable of neutralizing FMDV *in vitro*. The two peptides that elicited the highest neutralization titers were chosen for *in vivo* studies. Guinea pigs were injected subcutaneously with a single dose of peptide conjugate in either Freund's complete adjuvant or aluminum hydroxide gel (a medically acceptable adjuvant). Sera were harvested 35 days later and assayed for neutralization *in vitro*. As expected, the animals produced neutralizing responses. The guinea pigs were challenged with live virus by injecting  $10^4$  ID<sub>50</sub> of FMDV into one hind foot pad. The absence of the appearance of the typical foot-and-mouth disease lesions on the other feet of the animal was scored as protection against FMDV infection (25). Most animals were protected. Indeed, certain doses were as effective as the classical vaccine and as such this is the first demonstration that the synthetic peptide immunogen approach can generate an effective vaccine (25). We have since developed a vaccine that will protect against all three serotypes of FMDV (A, O, and C) by coupling peptides representing each serotype to the same carrier (F. Brown et al, in preparation). This suggests that a multicomponent vaccine could be generated by coupling peptides representing different pathogens to the same carrier. For example, a vaccine against FMDV and influenza virus could be made by coupling the above identified effective peptide immunogens to the same carrier protein.

### *Immunologic Therapy*

Neoplastic cells often express unique, cell-surface proteins—the so-called tumor or transformation specific antigens (reviewed in 57). By using antibodies directed against these antigens, one could specifically target the neoplastic cells for treatment (66). Indeed, monoclonal antibodies to certain tumor specific antigens have been isolated and are being readied for clinical trials. Synthetic peptide immunogens can fit into this approach as the immunogen for eliciting antibodies of the desired specificity, since it is often easier to clone and determine the nucleotide sequence of the gene that encodes the tumor-specific antigen than it is to produce monoclonal antibodies against it. The peptides can also be used as immunogens in the monoclonal hybridoma isolation procedures (H. L. Niman, R. A. Lerner, unpublished results), or can be used as an immunosorbent to purify the desired antibodies by immunoaffinity chromatography (97). Finally, an exciting future possibility is the prevention of certain types of cancer by vaccination with the appropriate synthetic peptide immunogen.

### *Designing a Vaccine*

When confronted with a novel pathogen or tumor cell, how can one generate a reagent for combating it? The following scenario, which combines immunolo-





gy, recombinant DNA technology, and the peptide immunogen approach, may produce antibodies that will neutralize the pathogenic organism or tumor cell. First, immunologic and serological studies are undertaken to identify which proteins are the targets of a neutralizing immune response. For example, the selection of VP1 of FMDV and hemagglutinin of influenza for our initial studies (25, 60) was because they were the major targets of neutralizing antibodies for each virus. In the absence of this information, it would be necessary to study all the proteins of a pathogen—obviously a formidable task. The next step is to determine the amino acid sequence of each protein identified by the immunologic studies. Currently the most straightforward way to do this is to clone the gene that encodes the neutralizing target and then determine the nucleotide sequence of the gene. The amino acid sequence is deduced from the nucleotide sequence. For many viruses and cDNA clones of mRNA, the amino acid sequence is colinear with the nucleotide sequence. For genomic clones of eukaryotic DNA, coding regions (exons) are often interrupted by noncoding regions (introns) and hence deduction of an amino acid sequence is more difficult.

With the amino acid sequence as a blueprint, peptides are chosen for synthesis and use as immunogens. The guidelines described above will be useful in selecting peptides that elicit protein-reactive antibodies, but not all peptides will give the desired immune response. Only 13 of the 26 influenza virus hemagglutinin peptides were capable of eliciting neutralizing antibodies (2, 42). The immunologic or serologic studies may be helpful in selecting neutralizing peptides. For example, a comparison of the amino acid sequences of the protein from different serotypes may identify a particular region of the protein as the target of the neutralizing antibody elicited by a natural infection. This exact consideration led to the proper region of the hepatitis B virus surface antigen (39, 40, 77, 85). Alternatively, one could adopt a brute force approach and simply make many or all of the peptides that fit the guidelines. This somewhat inelegant method is economically feasible and will identify the appropriate peptide. In addition, this approach may identify a peptide that can cross-neutralize several serotypes, as was the case for several of the influenza virus hemagglutinin peptides (2). Once an effective peptide is found, the optimal peptide corresponding to this region of the protein can be identified by analyzing a series of overlapping peptides. Finally, the appropriate peptide(s) or peptide-elicited antibodies must be developed for clinical applications. At this stage one must address questions regarding chemical purity, possible side effects or cross-reactivities, carrier, adjuvant, immunization schedule, route of administration, dose, etc. Most of these questions have not yet been addressed directly and obviously will be studied intensively as a synthetic peptide vaccine approaches the marketplace.



With respect to these medical applications, two relevant concerns are the dose of immunogen required to elicit a protective response and the specificity of the resulting antibodies. In our studies with FMDV (25), a 20- $\mu$ g dose of peptide in alum protected three of four guinea pigs from FMDV and a 200- $\mu$ g dose protected three of three animals, whereas a 1- $\mu$ g dose of killed virus protected only one of four animals and a 10- $\mu$ g dose protected two of two animals. Obviously, these studies are too limited to generate any solid conclusions, but in guinea pigs it appears that a 200- $\mu$ g dose of peptide may be as effective as a 10- $\mu$ g dose of the classical vaccine. In cattle, a single 1-mg dose is sufficient to protect against FMDV. With present small-scale technology, such a dose would cost about \$1, but the cost may drop by more than an order of magnitude with mass production and would probably drop further as attention turns to the organic chemistry involved. The optimal dose for a given peptide will need to be determined empirically since the generation of neutralizing antibodies is influenced by many factors, such as the immunogenicity of the particular peptide, its persistence after immunization, the carrier, the adjuvant, the route of administration, the immunization schedule, etc. Much work needs to be done especially in the area of carriers and adjuvants, though the efforts of Sela and co-workers (21a) with chemically synthesized adjuvants and carriers seems promising (see 9 for review).

The recent results of Nigg et al (68) raise the possibility that peptides may elicit unwanted reactivities—a serious problem for any medical application. Antibodies were raised against the carboxyl terminal hexapeptide of the transforming protein of Rous sarcoma virus. At low antibody and antigen concentrations, the antibodies specifically reacted only with the transforming protein. At very high antibody and antigen concentrations, the antibodies reacted specifically with the transforming protein and some other normal cellular components; three of which were identified as myosin,  $\beta$ -tubulin, and vimentin. Two explanations for this cross-reactivity are possible. First, the proteins could fortuitously share the peptide amino acid sequence. A given hexapeptide sequences should be present about once in every  $10^5$  proteins of 60,000 daltons. However, cross-reactivity based on shared sequences should occur at the same affinity, here reflected as reactivity at the same antibody and antigen concentrations. Thus, it seems unlikely that this consideration explains the cross-reactivity, and in the one case where the amino acid sequence of the peptide was compared to that of one of the cross-reacting proteins,  $\beta$ -tubulin (95), no identical matches were found. Furthermore, a peptide corresponding to a sequence found in a normal host protein probably would not generate protein-reactive antibodies due to tolerance mechanisms. For example, Jemmerson et al (48) are studying possible tolerance mechanisms by using a peptide immunogen that corresponds to residues 41–49 of mouse cytochrome c. Im-



munization of mice with the decapeptide produced antibodies that reacted with the peptide but not the native protein. Mouse pre-B cells could respond to the peptide and bind to the native protein, but mature B-cells secreting protein-reactive antibodies were not found. This suggests that in this case, tolerance acts by eliminating a specific population of B cells.

The cross-reactivity with the normal cellular proteins occurs at  $10^3$ – $10^4$  lower affinity than reactivity with the transforming protein. Monoclonal antibodies exhibit similar lower affinity binding to nontarget proteins (33, 37, 55, 73). In fact, monoclonal anti-thy 1.1 cross-reacts with vimentin (37) and monoclonal anti-thy 1.2 cross-reacts with actin (S. Dales, personal communication). Such cross-reactivity is not surprising since antibodies obey mass action laws, and these additional targets are present at high concentrations in cell extracts as they are major cell structural proteins. (Low affinity binding will be detected when the product of the antibody and antigen concentrations is high.) Antigenic sites that resemble the target antigenic site by matching four of the six amino acids of the hexapeptide may exhibit low affinity binding. For example, Nigg et al (68) suggest that the  $\beta$ -tubulin binding is due to the sequence Val-Leu-Asp-Val-Val-Arg (target sequence is Val-Leu-Glu-Val-Ala-Glu). Such similar sequences (a three of six match) should be present about once in a thousand 60,000-dalton proteins. Obviously, these cross-reactivities are important in vitro since the experimenter can adjust the antibody and antigen concentration. But in vivo, the required high concentrations of antibody and antigen are unlikely and probably will not lead to unwanted side effects in synthetic peptide vaccines.

## PEPTIDE IMMUNOGENS IN BASIC RESEARCH

The uses of peptide immunogens and the resulting protein-reactive antibodies have been recently reviewed (59, 90) and we only briefly discuss the two facets of the approach that made it particularly useful in basic research. First, essentially all one needs to produce antibodies that react with a given protein is its amino acid sequence. Although a few years ago such a statement would seem paradoxical since most amino acid sequence was being generated from analysis of purified proteins, and if one had purified protein, it was a simple step to generate a specific antiserum, nowadays most amino acid sequence is generated by translation of nucleotide sequences. Indeed, occasionally all one knows about a putative gene product is its deduced amino acid sequence. The protein-reactive antibodies are excellent reagents for identifying (22, 82, 91), localizing (68), and purifying (97) the protein product and analyzing possible enzymatic activities (23). The second facet is that the peptide-elicited antibodies react with a small region of the protein that is chosen in advance by the investigator. Thus, the antibodies are reagents for following the fate of particu-

lar portions of the protein through protein processing pathways. This fine-specificity of binding can also be exploited in the analysis of DNA or RNA rearrangements that alter protein sequence such as occurs during immunoglobulin production (1, 31). In toto, the peptide immunogen approach provides a powerful set of reagents of predetermined specificity with which to investigate protein expression, processing, localization, and activity.

## SUMMARY

Synthetic peptide immunogens have been shown to elicit antibodies that can react with full-length proteins containing that peptide. Such antibodies are directed against a specific region of the protein chosen in advance by the investigator and so have a predetermined specificity. In basic research, these antibodies are useful in identifying the protein product of an open reading frame, localizing the gene product to particular cells or subcellular organelles, identifying the enzymatic function of a protein product, following the fate of particular regions of a product through protein maturation processes, analyzing the expression of exons following DNA rearrangements and RNA splicing, and purifying the protein by immunoaffinity chromatography techniques. In medicine, such antibodies may provide reagents for passive vaccination, antitoxin therapy, and targeted immunotherapy of neoplasia. The peptides themselves may be used as synthetic vaccines.

The immediate future of the synthetic peptide immunogen in medicine is clear—the promise demonstrated in the laboratory must be reduced to safe application in the hospital. Two barriers to this are the selection of precisely the best peptide and the selection of the proper adjuvant. Currently, a brute force approach is utilized to find the best peptide for eliciting the desired antibodies. This is clearly a problem when the pathogenic organism is assayable only in man. Possibly, by combining studies on the antigenicity of the pathogenic organism with an analysis of naturally occurring variants that alter its immunogenicity, peptide selection will be made easier. Also, since the adjuvants and carriers used in the laboratory are in general too harsh for widespread use in humans and animals, much work needs to be done to find suitable adjuvants and carriers. Nonetheless, now that the major conceptual hurdle to synthetic peptide vaccines has been cleared (that is, it is not necessary to reproduce conformation exactly), it should be relatively straightforward to solve the remaining problems.

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